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Jean C. Baker

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Appl. No.: | 09/970,382 Client Docket No.: P01258US |
| Applicants: | Su-Chun Zhang, et al. |
| Filed: | October 3, 2001 |
| Title: | METHOD OF *IN VITRO* DIFFERENTIATION OF TRANSPLANTABLE NEURAL PRECURSOR CELLS FROM PRIMATE EMBRYONIC STEM CELLS |
| TC/A.U.: | 1636 |
| Examiner: | Q. Nguyen |
| Docket No.: | 960296.98211 |

Honorable Commissioner for Patents
Washington, DC 20231



**DECLARATION OF DR. SU-CHUN ZHANG**

Sir:

I, Su-Chun Zhang, declare that:

1. I am a named inventor of the above-identified case. I am a faculty member at the University of Wisconsin, Madison in the Department of Anatomy and Neurology, Stem Cell Research Program. I have been asked to comment by Attorney Jean C. Baker on the Examiner's characterization of the Carpenter, et al. application in the Examiner's November 19, 2002 Office Action.

2. The cells created by the Carpenter method and the cells created by the method disclosed in the Zhang, et al. application differ in objective, differentiation medium, and outcome. I have summarized some of these differences in Table 1, attached to this statement.

Considered by Q.N. 6/17/03

3. I have also attached "Mammalian Neural Stem Cells", *Science* 287, 25 Febraury 2000, 1433-1438 as containing an illustration identifying various classes of mammalian stem cells that can give rise to neurons.

4. Both the Carpenter group and my group use the same starting cell source, human embryonic stem cells generated by the Thomson method. Carpenter's objection is to obtain neural progenitor cells that are restricted to either neurons or glial cells (see definition on line 10, page 6; line 39-43 of page 11, also claims 1-4 on page 30). The objective of my method is to obtain a synchronized population of neural precursor cells, or neuroepithelial cells, also known as neural stem cells. (By definition, neural stem cells are those that can give rise to neurons, astrocytes, and oligodendrocytes.)

5. The initial stage of differentiation is the formation of embryoid body in suspension culture. However, in Carpenter's protocol, 10 uM of retinoic acid was added to the culture (page 386 of the publication, or page 11, line 34-35 of patent) whereas in my method, there was no retinoic acid. Retinoic acid is a strong morphogen. A high amount of retinoic acid is mutagenic in human development. It has been used to promote neural differentiation from mouse ES cells using 1 uM. However, use of retinoic acid strongly promotes differentiation. That explains why in only 3 days there were

already neurons in the Carpenter method (way beyond neural progenitor stage).

6. The base differentiation medium is the same, DMEM/F12. However, the cocktail is substantially different. In biology, it is not always better to have many growth factors than a single factor. This is particularly true when a group of conflicting factors in the same mixture do a single job. FGF2 is an inducing and proliferating factor for neural stem cells. EGF is a proliferating factor for neural stem cells. PDGF and IGF-1 are differentiating factors, promoting neuronal differentiation (maturation) from neural stem cells. Hence, under the influence of these contradicting factors, the Carpenter culture ended up with a mixture of neural cells from progenitors to mature neurons. In contrast, in my protocol treatment with a single factor, FGF2, induces a synchronized population of neural precursor cells without the presence of mature neurons. FGF2 is a well-known neural-inducing and neural-proliferating factor in chick.

7. It is also worth noting that my protocol results in the generation of neural precursor (or stem) cells in exact timing as it should be in normal human development. In Carpenter's protocol, neurons appear in only 3 days of culture, substantially earlier than normal development. That suggests that the normal developmental program has been altered. This can be explained by the treatment with the morphogen retinoic acid and a group of differentiating factors

such as PDGFaa and IGF1. Hence, shorter protocol with more factors does not mean better. In practice, a simple protocol that is more akin to normal development would be better.

8. Because of the different treatments, the outcome is substantially different. Carpenter's protocol yields a mixture of neural cells from progenitors to mature cells whereas my protocol leads to a synchronized neural precursor population that organizes into a neural tube-like rosette formation (see Fig. 1 of Attachment #2). Rosette formation is the feature of neuroepithelial cells (earliest neural cells). It has only been reported in mouse ES cell-generated neural precursor cells using FGF2 when they were transplanted into embryonic mouse brain (Brustle, et al., 1997) and in terotomas formed by primate ES cells (Thomson, et al., 1995). Neural cells differentiated from Carpenter's protocol do not form rosette structures.

9. The Examiner has commented on the use of PSA-NCAM as a marker of a neural precursor cell. In the paragraph below, I explain that expression of PSA-NCAM is not indicative of a particular developmental stage of a cell.

10. Neural epithelial cells, or neural stem cells, are the earliest neural cells during development. They express nestin and PSA-NCAM (PSA-NCAM is expressed at a slightly later stage). During development, neural stem cells differentiate to give rise to intermediate cells called neural progenitor cells (see Fig. 1 of Attachment #2), such as neuronal

progenitors that give rise to only neurons, and glial progenitors that generate only glial cells (which is the same as the definition of Carpenter's). Expression of PSA-NCAM expands from neural stem cell stage to immature neuronal stage. Hence, the developmental stage of a cell cannot be defined simply based on the expression of PSA-NCAM. In fact, cells sorted by PSA-NCAM generate only neurons, implying that these cells are at the progenitor stage. Similarly, A2B5 has been shown to be expressed by human neurons, astrocytes, glial progenitor cells, and neuronal progenitor cells (Sato, et al., 1985). Hence, A2B5 sorted cells co-express neuronal markers b-tubulin and MAP2 and some of them co-express GFAP. Therefore, neural cells generated by Carpenter are at a later developmental stage, either neuronal or glial progenitors.

11. In summary, in Carpenter's protocol, exceedingly high concentration of retinoic acid and a mixture of conflicting growth factors yield a mixture of neural cells from progenitors to mature cells. In my approach, a single growth factor leads to synchronized differentiation of neural precursor cells (neuroepithelial cells) organized into a neural tube-like rosette.

12. In Carpenter's method, magnetic sorting with PSA-NCAM or A2B5 yields either neuronal progenitor cells or glial progenitors. This was their objective. The sorted cells do not generate oligodendrocytes although the mix culture contains GC positive cells (putative oligodendrocytes). Only

neural stem cells (or neuroepithelial cells) generates neurons, astorcytes, and oligodendrocytes. In my protocol, neural precursors in the rosettes are isolated by treatment with low concentration of an enzyme (dispase) which leaves non-neural cells behind, and differential adhesion in which non-neural cells adhere first whereas neural cells adhere more slowly. Hence, the neural precursors induced by FGF2, displayed by expression of nestin and PSA-NCAM, organized into rosette formation, are at an earlier developmental stage and have a broader differentiation potential.

13. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Respectfully submitted,

Dated: 4/10/03

Su-Chun Zhang